DESCRIPTION

MONITOR PROTEIN FOR MEASURING PROTEIN PHOSPHORYLATION

Technical Field

The present invention relates to a protein for measuring protein phosphorylation and a nucleic acid encoding the protein.

Background Art

Ingenious switch mechanisms are present in living organisms, and these switch mechanisms control, for example, differentiation, development, protective responses, metabolic activity, etc. A known switch mechanism is the phenomenon of protein phosphorylation (Hunter T., 1991, Methods Enzymol. 200: 3-37). Phosphorylation is mediated by an enzyme. The enzyme introduces a phosphate group to a substrate protein and activates the substrate protein. The activated substrate protein induces diverse reactions, for instance, regulation of gene expression and cellular proliferation.

Recently, a cascade mechanism, called cellular signal transduction, has been uncovered. Cellular signal transduction is the basis for diverse reactions in a living body, including cellular development and differentiation. The phosphorylation reaction has been shown to play an important role as one of the switch mechanisms which determine the on-state and off-state of the cellular signal transduction pathway (Hunter T., 1995, Cell 80: 225-236).

So far multiple pathways have been identified as signal transduction pathways. For instance, a relatively simple pathway that is a signal transduction pathway is one in which extracellular information, such as a hormone, directly passes through a cell membrane and is transmitted into the inside of a cell. More recently, complex

pathways through which information is transmitted into a cell by communication along multiple molecules have been discovered.

An example signal transduction pathway is mediated by a G protein (Gilman,.A. G. , 1987, Annu. Rev. Biochem. 56: 615-649). A signal for initiating the transduction pathway is binding of a specific substance to a receptor on a cell membrane. G protein conjugated to the receptor in a cell then elevates the intracellular concentration of cyclic-adenosine mono phosphate (cAMP), a second messenger. A specific kinase protein phosphorylates A kinase using the increased concentration of cAMP. It is proposed that phosphorylation of A kinase generated in this manner, namely by activation, is transmitted to, for example, the nucleus, as a signal, inducing expression, such as for a protein necessary for an *in vivo* response at the final stage of the cascade. Other signal transduction pathways are known and include, for instance, a transduction pathway based on a cell surface receptor and MAP kinase (Madhani, H. D. et al., 1998, Trends Genet. 14: 151-155) and a cell membrane phospholipid and C kinase (Weinstein, I. B. et al., 1997, Adv. Exp. Med. Biol. 400A: 313-321) indicating that protein phosphorylation is important as a switch that activates a pathway in all cases. Many studies have suggested, however, that the presently known signal transduction pathways are only part of the diverse transduction pathways expected to be present in a cell.

Thus, uncovering signal transduction pathways is important to reveal the various mechanisms of activities in life. Substances involved in novel and known transduction pathways have been searched for and the functions of these substances have been analyzed. Studies of signal transduction pathways have included analyses of the phosphorylation reaction as playing an important role as a switch mechanism in signal transduction, searches for enzymes that participate in this reaction, and such.

Enzymes that perform protein phosphorylation reactions have mainly been searched by comparing homology of known protein kinases with other genes. A consensus sequence has been identified in the family of protein kinases that phosphorylates a tyrosine residue (Hunter T. et al., 1984, Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17: 443-455). Thus, libraries can be screened using a probe based on this consensus sequence. An enzyme newly discovered in this manner is analyzed for protein phosphorylation activity mainly by *in vitro* analysis using ^{32}P (D. Grahame Hardie, Eds. "Protein Phosphorylation: A Practical Approach," 1993, Oxford University Press). Specifically, a searched enzyme, a substrate protein (or a known peptide) comprising phosphorylation region, and ~~a~~ ^{32}P phosphate are mixed to determine whether the ^{32}P is added to the substrate protein (or the peptide), by monitoring radioactivity of a reaction product.

Conventional methods for analyzing protein phosphorylation require the use of radioactive isotopes, such as ^{32}P, limiting experimental facilities and such, requiring careful experimental manipulation, and thus making manipulation cumbersome. In addition, these kinds of experiments generate radioactive waste, and therefore, are not preferable for the natural environment. Moreover, the above-described analytical method does not directly measure intracellular phosphorylation activity, and thus, is problematic because it only provides results measured in an artificial environment.

To solve these problems an *in vivo* measurement system was developed. The method did not directly measure phosphorylation. The method measured protein phosphorylation indirectly using cellular proliferation induced by phosphorylation as an indicator. More specifically, in this analysis system ^{3}H thymidine uptake into a cell measured the cellular proliferation rate. Based on the cellular proliferation rate,

phosphorylation at an early stage in the signal transduction pathway was measured. In this method it is difficult to rapidly measure phosphorylation because an activity at the end of transduction, cellular proliferation, is measured as an indicator. Moreover, the method is not suitable for measuring the timing of the phosphorylation reaction and such.

An analysis system that measures calcium concentration *in vivo* has recently been reported (Miyawaki, A. et al., 1997, Nature 388: 882-887). This system uses a fusion protein. The fusion protein contains green fluorescent proteins (GFP) of luminous jellyfish, *Aequorea victoria,* fused to both ends of a calmodulin protein. Fluorescence is emitted when the GFP proteins at the both ends approach and interact with each other. Thus, when a certain amount of calcium is bound to the central calmodulin protein, the conformation of the calmodulin protein is altered, and the GFP proteins at the both ends interact with each other to emit fluorescence. *In vivo* calcium concentration is determined by measuring the emitted light. Thus, a superior method that measures calcium concentration which does not use radioactive isotopes and is applicable to the *in vivo* measurement, has been developed. However, in the above-described protein phosphorylation reaction, such a system has not been developed.

Disclosure of the Invention

An objective of the present invention is to establish a system to analyze protein phosphorylation, in which radioactive isotopes are not used and that is applicable to *in vivo* measurement.

The present inventors first obtained genes encoding the cAMP response element binding protein (CREB) phosphorylation sequence (Gonzalez, G. A. et al., 1989, Cell 59: 675-680) and the kemptide phosphorylation sequence (Kemp B. E. et

al., 1977, J. Biol. Chem. 252: 4888-4894), which are representative examples of known amino acid sequences that are phosphorylated. The genes were inserted between a red-shifted green fluorescent protein (RSGFP) site (also called RGFP) and a blue-shifted green fluorescent protein (BSFGP) site (also called BGFP) of a plasmid pETIC (Romoser V. A. et al., 1997, J. Biol. Chem. 272: 13270-13274) to prepare plasmids "pETIC-ART" and "pETIC-Kempart," respectively. Both plasmids can express a fusion protein comprising "RSGFP- a protein phosphorylation sequence-BSGFP" and are different only in the protein phosphorylation sequences. As a negative control in the phosphorylation reaction experiment, pETIC-1 was used. This plasmid can express a fusion protein comprising "RSGFP- a calmodulin binding site - BSGFP" (Romoser V. A. et al., J. Biol.Chem., 1997, 272: 13270-13274).

E. coli BL-21 (DE3) was transformed with pETIC-ART, pETIC-Kempart, or pETIC-1, and the above fusion proteins were extracted from the microbial cells after incubation.

Analysis using an *in vitro* analysis system with ^{32}P isotope found that the fusion proteins derived from pETIC-ART and pETIC-Kempart were phosphorylated, but the fusion protein derived from pETIC-1 was not.

A change in fluorescence was determined using a non-radioactive phosphate as a substrate. The fusion protein derived from pETIC-ART showed differences in absorption wavelength depending on whether phosphorylation occurred with the nonradioactive phosphate. The fusion proteins derived from pETIC-Kempart or pETIC-1 did not show differences in absorption wavelength depending on whether phosphorylation occurred. These results uncovered the following two points.

1. Phosphorylation of the fusion protein derived from "pETIC-ART" generates protein conformational change necessary for generating changes in GFP fluorescence.

2. The fusion protein derived from "pETIC-Kempart" is phosphorylated, however, this phosphorylation does not generate protein conformational change necessary for generating changes in GFP fluorescence.

Specifically, the present inventors have shown that, in some cases, protein phosphorylation generates a conformational change in a protein. Using the combination of two kinds of GFP proteins, as described above, provides an example for detecting conformational change as a property change. Thus, it is obviously possible that the same system can be constructed for proteins having other properties, etc.

Thus, fusion of a protein containing a region for monitoring a property change, a variable property region, and a region to be phosphorylated, a phosphorylation region, can be used as a protein for monitoring protein phosphorylation (a monitor protein). The use of a monitor protein does not require the use of radioactive isotopes and is applicable to *in vivo* measurement. In fact, the present inventors have successfully monitored phosphorylation in real time in living cells into which a vector expressing a monitor protein has been introduced.

Such a monitor protein can easily monitor protein phosphorylation, and therefore can be used in a method for screening for a novel kinase and such. Moreover, using this system, a compound which stimulates or inhibits phosphorylation can be screened.

The finding that some protein kinases generate a conformational change in a substrate, and others do not indicates that monitor proteins can also be used to

determine whether protein phosphorylation generates a conformational change. The methods would use both an existing *in vitro* analysis system with ^{32}P isotope, and a method using the property change of a monitor protein. More specifically, the present invention relates to:

(1) a monitor protein for measuring protein phosphorylation, the monitor protein comprising (a) a phosphorylation region comprising an amino acid residue or an amino acid sequence to be phosphorylated, and (b) a variable property region showing a property change attributed to a conformational change of a protein comprising at least the phosphorylation region, which conformational change is caused by phosphorylation of the amino acid residue or the amino acid sequence;

(2) the monitor protein of (1) , wherein the variable property region is a protein that emits fluorescence;

(3) the monitor protein of (1) or (2), wherein the variable property region is bound to each of both ends of the phosphorylation region;

(4) the monitor protein of (3), wherein the variable property region comprises RSGFP and BSGFP which are comprised in green fluorescent protein (GFP) of *Aequorea victoria*;

(5) the monitor protein of any one of (1) to (4), wherein the phosphorylation region comprises the amino acid sequence of SEQ ID NO: 1

(6) a nucleic acid encoding the monitor protein of any one of (1) to (5);

(7) an expression vector carrying the nucleic acid of (6);

(8) a method for measuring phosphorylation ability in a cell by introducing the monitor protein of any one of (1) to (5), the nucleic acid of (6), or the expression vector of (7) into the cell;

(9) a method for measuring phosphorylation ability of a test protein, the method comprising reacting the test protein with the monitor protein of any one of (1) to (5), and measuring a property change of the monitor protein;

(10) a method for screening a kinase, the method comprising:

(a) reacting a test protein with the monitor protein of any one of (1) to (5),

(b) measuring the property change of the monitor protein, and

(c) selecting the test protein which alters the property of the monitor protein;

(11) a method for screening a compound which stimulates or inhibits phosphorylation, the method comprising:

(a) contacting, in the presence of a test sample, a kinase with the monitor protein of any one of (1) to (5), the monitor protein comprising a phosphorylation region to be phosphorylated by the kinase,

(b) measuring the property a change of the monitor protein, and

(c) selecting a compound which stimulates or inhibits the property change in comparison with the property change in the absence of the test sample; and

(12) a method for screening a compound which stimulates or inhibits phosphorylation, the method comprising:

(a) preparing a cell into which the expression vector of (7) is introduced,

(b) measuring, in the presence of a test sample, the property change of a monitor protein expressed in the cell, and

(c) selecting a compound which stimulates or inhibits the property change in comparison with the property change in the absence of the test sample.

The present invention is illustrated in detail below.

1. A monitor protein for measuring protein phosphorylation

A monitor protein for measuring protein phosphorylation (a monitor protein, hereafter) is a protein comprising a "phosphorylation region" and one or more "variable property regions."

Herein, a "phosphorylation region" means a region comprising an amino acid residue to be phosphorylated and capable of changing its conformation by phosphorylation of the amino acid residue. Among the proteins known to be phosphorylated, those capable of showing conformational change by phosphorylation can be used as a phosphorylation region. As the protein to be phosphorylated, exemplary examples are CREB transcription factor (Hagiwara M. et al., 1993, Mol. Cell. Biol. 13: 4852-4859) and ATF1 (Shimomura, A. et al., 1996, J. Biol. Chem. 271: 17957-17960).

A conformational change can even be induced in a protein, for example, which does not usually undergo any conformational change by a phosphate group-specific antibody. Therefore, a phosphate group-specific antibody can be combined with another property variable region.

The phosphorylation region is preferably a partial sequence of a protein comprising an amino acid residue to be phosphorylated but can also be the full length protein. For instance, in the case of CREB transcription factor, it is known that the serine residue at amino acid 133 is phosphorylated by protein kinase A. Therefore, any partial sequence of CREB transcription factor can be used as long as it contains the serine residue at amino acid 133 and is capable of being phosphorylated. For

example, as the partial sequence, an amino acid sequence of SEQ ID NO: 1 can be selected. As an "amino acid residue to be phosphorylated," for example, tyrosine, threonine, and so on can be used other than serine.

Any amino acid sequence can be used as a "variable property region" as long as its phosphorylation can be easily determined. An example is GFP.

"Property variable regions" can be separately present as long as they form a fusion protein with a phosphorylation region. A monitor protein can preferably be constructed as a "measurement protein pair" provided at both ends of a phosphorylation region. More specifically, an amino acid residue in a phosphorylation region is phosphorylated to alter the conformation of the phosphorylation region, and a measurement protein pair prepared at both the ends of the phosphorylation region interacts to show a measurable property. For example, each component can be designed as follows.

Any "measurement protein pair" provided at both ends of the above phosphorylation region can be used as long as the pair displays a measurable property after interaction due to conformational change of the phosphorylation region. For example, proteins emitting fluorescence by interacting with each other, such as BSGFP and RSGFP of *Aequorea victoria*, can preferably be used.

Any desirable functional regions can be added to a monitor protein other than the above "phosphorylation region" and "variable property region." Examples include a nuclear localization signal to transport a monitor protein to the nucleus and measure phosphorylation in the nucleus (Goldfarb, D. S. et al., 1986, Nature 322: 641-644) and a marker as an indicator that a monitor protein is introduced into a cell (Heim, R. et al., 1995, Nature 373: 663-664).

2. Production of a monitor protein

Any methods for producing the above monitor protein can be used. For example, a monitor protein can be produced by expressing a nucleic acid encoding the monitor protein in any host cell, such as *E. coli*. More specifically, this "nucleic acid encoding a monitor protein" is constructed so that a measurement protein pair and a phosphorylation region can be expressed as a fusion protein. The nucleic acid is produced by ligating a nucleotide sequence encoding the above variable property region and a nucleotide sequence encoding the phosphorylation region in the same reading frame. A monitor protein can be amplified and produced by ligating the nucleic acid encoding the monitor protein constructed above to any vector, introducing the vector into an appropriate host cell, and expressing the nucleic acid. A monitor protein produced in this manner can be used for measuring *in vitro* or *in vivo* phosphorylation directly, or preferably be used after isolation and purification.

Any combination of a vector and a host cell can be used for producing a monitor protein, and the nucleic acid encoding a monitor protein can be ligated downstream of a promoter with high expression activity for improving monitor protein expression. Such a promoter and the like can be arbitrary selected from known promoters and used.

3. A nucleic acid encoding a monitor protein

The above "nucleic acid encoding a monitor protein" can include other sequences as long as the nucleic acid encodes the monitor protein. These other sequences include a sequence that causes effective expression of a monitor protein in a cell, for example, a regulatory sequence, such as a promoter and enhancer; a selective gene, such as a drug resistance marker for detecting introduction of the nucleic acid into cells; etc.

This nucleic acid can be used for directly detecting a phosphorylation reaction in a cell by introducing the nucleic acid into the cell and directly expressing the above monitor protein as well as for producing the monitor protein. The monitor protein can be transported into a cell more effectively by introducing the nucleic acid encoding the monitor protein into the cell, because, in general, nucleic acids can be introduced into a cell more effectively than proteins. In addition, as described later, the above nucleic acid can be easily transported into a cell and prepared by harboring the nucleic acid in a vector.

4. A vector carrying a nucleic acid encoding a monitor protein

Any vector can be used. as long as it is capable of self-replication and contains a transcription initiation sequence. Any known plasmids which can be expressed in, for example, *E. coli*, yeast, plant cells, insect cells, mammalian cells, and such can be used. A plasmid can be selected from these known plasmids, as well as a corresponding cell.

5. A monitor protein for screening a kinase

A monitor protein can be used to search and to screen for an enzyme which phosphorylates a phosphorylation region in the monitor protein. An enzyme which phosphorylates a phosphorylation region can be screened by reacting a monitor protein comprising the desirable phosphorylation region and a test protein *in vivo* or *in vitro* and detecting phosphorylation of the monitor protein. In the case of screening *in vivo*, a vector carrying a nucleic acid encoding a monitor protein can be used. Any test proteins can be used. Natural protein libraries, artificial protein libraries, cDNA libraries thereof, and such can be used. Alternatively, for example, ribozyme libraries can be used.

6. A monitor protein for screening a compound which stimulates or inhibits phosphorylation

A monitor protein can be used for screening an accelerator or inhibitor of phosphorylation or dephosphorylation enzyme activity. For example, a desired kinase is contacted with a monitor protein comprising the phosphorylation region to be phosphorylated in the presence of a test sample. A property change of the monitor protein is detected. Any test sample can be used. Examples of a test sample include cellular extracts, expression products of gene libraries, synthetic low molecular compounds, synthetic peptides, modified peptides, natural compounds. A compound which stimulates or inhibits a property change is screened by comparing with a property change in the absence of the test sample. A compound which accelerates a property change of the monitor protein is identified as a compound which stimulates kinase activity, and a compound which inhibits a property change of the monitor protein is identified as compound which inhibits kinase activity. This enables isolation of a compound which stimulates or inhibits phosphorylation activity of the kinase.

Combinations of a kinase to be used for screening and a phosphorylation region are not particularly limited.

Examples of kinases used for screening in the present invention (and phosphorylation sequences thereof) include, but are not limited to, A kinase (CREB phosphorylation sequence of SEQ ID NO: 1), G kinase (RKRS*RAE; histone), AMP activation protein kinase (HMRSAMS*GLHLVKRR; acetyl CoA carboxylase), calmodulin-dependent protein kinase II (PLRRTLS*VAA; glycogen synthase) smooth muscle myosin light chain kinase (KKRAARATS*NVFA; myosin light.chain), phosphorylase kinase (KRKQIS*VRGSL; phosphorylase), C kinase (VRKRT*LRRL; EGF receptor), v-Abl (RRLIEDAEY*AARG; $RR60^{SRC}$), and EGF receptor protein kinase (RREELQDDY*EDD; erythrocyte band III) (R. B. Rearson et al., "Specific studies on protein kinase and phosphatase using synthetic peptides"; D. G.. Hardie Eds., supervised by Hidaka, H., MEDSi Biological Experiment Series, Protein Kinase and Phosphatase, pp225-228, 1995, Medical Science International). Sequences and proteins in parentheses are substrate amino acid sequence and substrate proteins to be phosphorylated. These kinases are known to phosphorylate proteins comprising an amino acid sequence other than the sequences identified above. Therefore, an amino acid sequence at these substrate sites can be appropriately selected and used.

In the case of screening *in vivo*, a vector carrying a nucleic acid which encodes a monitor protein can be used. In this system, for example, compounds which stimulate or inhibit signal transduction leading to expression and activation of the kinase, and compounds which stimulate or inhibit dephosphorylation of a monitor protein as well as compounds which directly stimulate or inhibit the activity of the target kinase (an agonist and antagonist of the kinase) can be screened. Whether a

compound directly acts on a kinase or not can be confirmed by an *in vitro* system using a purified kinase. In an *in vivo* system, for example, in the case of using GFP as a variable property region as described in Examples, property change of a monitor protein can be detected without crushing cells. Moreover, a property change of the monitor protein can be detected in real time in living cells. Effects of a test compound on phosphorylation can be examined in detail in this manner.

These screenings, for instance, can conveniently screen for an inhibitor specific to a particular kinase. Kinases have important functions in signal transduction in, for example, cellular proliferation, differentiation, and immunological responses. Thus, an isolated inhibitor is expected to be able to be applied as a drug for preventing or treating various diseases involved in signal transduction.

Brief Description of the Drawings

Figure 1 shows the construction of pETIC vector.

Figure 2 shows. the purified recombinant protein separated by SDS-PAGE and stained with CBB.

Figure 3 shows the results of phosphorylation assay by A kinase.

Figure 4 shows the effect of the addition of A kinase on absorption wavelength for "A-Kinase Responsive Tracer (ART)" (derived from pETIC-ART).

Figure 5 shows the effect of addition of A kinase on absorption wavelength for "Kempart" (derived from pETIC-Kempart).

Figure 6 shows the effect of addition of A kinase on absorption wavelength for a negative control protein (derived from pETIC-1).

Figure 7 shows the construction of pCEP4.

Figure 8 shows the figure of ART in COS-7 living cells. (A) shows a BSGFP fluorescence image of ART expressed in a whole cells. (B) shows pseudo color images of BSGFP/RSGFP fluorescence intensity ratio in the cells in the same region as (A). The cells were treated with 5 μM dibutylyl-cAMP (db-cAMP) at hour 0. The bar indicates 30 μm.

Figure 9 shows the fluorescence intensity ratios of ART in COS-7 cells (mean±SEM, n=5) after the treatment with db-cAMP in the presence of H-89, which is a PKA inhibitor (the white bars), or the absence thereof (the black bars). Dibutylyl-cAMP (db-cAMP), which is a cAMP analogue, was added at hour 0. R indicates the florescence intensity ratio of cellular images detected in the region of about 10 μm X 10 μm square at the various times. Rmin indicates the minimum fluorescence intensity ratio of the same cells.

Best Mode for Carrying Out the Invention

The present invention is illustrated in detail below with reference to the Examples, but is not construed as being limited thereto.

In these Examples, phosphorylation was detected by constructing a monitor protein, A-kinase Responsive Tracer ("ART"). The ART contained the phosphorylation sequence of CREB transcription factor (referred to as CREB phosphorylation sequence hereafter) as a phosphorylation region and RSGFP and BSGFP of *Aequorea victoria* as a measurement protein pair. SEQ ID NO: 1 shows the CREB phosphorylation sequence.

A monitor protein "Kempart" was constructed in the same manner using the phosphorylation sequence (SEQ ID NO: 2) of kemptide instead of the CREB

phosphorylation sequence. The kempart monitor protein was compared to the monitor protein containing the above CREB phosphorylation sequence.

Example 1: Synthesis of a DNA fragment encoding a phosphorylation sequence

First, a DNA fragment encoding a phosphorylation sequence was synthesized to construct the above monitor protein. A DNA fragment encoding the CREB phosphorylation sequence (refer to as the CREB-DNA fragment, hereafter) was amplified by PCR using oligonucleotide LCR-1B (SEQ ID NO: 3) as a template, and PCR-1K (SEQ ID NO: 5) and PCR-1A (SEQ ID NO: 6) as primers.

A DNA fragment encoding the phosphorylation sequence of kemptide (referred to as kemptide-DNA fragment hereinafter) was used as a control and was similarly amplified by PCR using oligonucleotide Lke-1B (SEQ ID NO: 4) as a template, and PKe-1K (SEQ ID NO: 7) and PKe-lA (SEQ ID NO: 8) as primers. Detailed manipulation by PCR is as follows.

The PCR reaction mixture was prepared in a microtube (for 0.2 ml). The composition of the PCR reaction mixture was sterilized water (18.3 μl), 10 X EXTaq buffer (2. 5 μl), dNTP mixture (2. 0 μl), EXTaq polymerase (0. 2μl) (Takara), template oligonucleotides (2 0 nmol/ml) (1.0 μl), and primers (about 50 nmol/ml) (0.5 μl each).

A microtube containing the above reaction mixture was set on a DNA amplifier (GeneAmp PCR System 2400, Perkin Elmer Japan) and amplified by PCR. PCR was performed under the following conditions: 1 cycle of a denaturation process (94°C for 30 sec); 40 cycles of a series of a denaturation process (94°C for 50 sec), an annealing process (57°C for 1 min), and an extension process (72°C for 1 min); a final extension process (72°C for 7 min); and cooling process at 4°C.

After PCR, amplified fragments were confirmed by electrophoresis. A portion of the reaction mixture (10 μl) was collected for electrophoresis and mixed with an electrophoresis buffer. This mixture was separated on a 10% agarose gel and stained with ethidium bromide to confirm the presence of target fragments.

The target fragments were excised with a razor and such, transferred to a microtube (for 1.5 ml), and recovered using Geneclean III kit (BIO 1). Each collected DNA target fragment was dissolved into Tris EDTA (TE) buffer (100 μl) and then purified. Purification was performed by the following procedure. First, a mixture of phenol, chloroform, and isoamyl alcohol (25: 24: 1) was added to the DNA solution and stirred. After the solution was left to stand, the supernatant was removed, and ethanol precipitation was conducted. The supernatant was removed, and precipitated pellet was dissolved in sterilized water.

Example 2: Construction of a plasmid carrying a gene of a monitor protein

Each of the above DNA fragments was inserted into the pETIC vector (J. Biol. Chem., 272: 13270-13274, 1997) shown in Fig. 1. The pETIC vector contains the RSGFP gene and BSGFP gene as a measurement protein pair. Thus, a monitor protein gene which contains a "RSGFP gene - a phosphorylation sequence - BSGFP gene" was constructed by inserting the above DNA fragments between the RSGFP and BSGFP gene.

First, the pETIC vector and the above two DNA fragments were digested with restriction enzymes KpnI and AgeI according to the standard method. After digestion, each of the fragments and the linearized-vector were separated on a 10% agarose gel by electrophoresis. After separation, each target fragment was collected with

Geneclean III kit in the same manner as described above and purified. Each of these fragments was dissolved in TE buffer (10 μl).

Next, CREB-DNA fragment or kemptide-DNA fragment was ligated to pETIC vector using the above DNA solution. Ligation was conducted with a DNA Ligation Kit Ver. 2 (Takara).

The CREB fragment solution (5 μl) was mixed with the linearized pETIC vector solution (3 μl). Solution I (8 μl) of the above kit was added thereto and the reaction was performed at 16°C for 30 min. The kemptide fragment solution (5 μl) was mixed with the pETIC vector solution (3 μl) and solution I (8 μl) of the above kit to perform ligation reaction in the same manner. The ligation was also prepared using TE buffer instead of the insert.

To collect a target plasmid from the above ligation reaction solution, the above ligation reaction solution (8 μl) was mixed with JM109 competent cells (100 μl). Transformation was conducted according to the standard method. After transformation, the JM109 cells were spread on an agar medium containing kanamycin and cultured at 37°C overnight. After incubation, kanamycin-resistant colonies that grew were taken, and the plasmids in the cells were analyzed. Cells containing plasmids in which the target fragments were inserted into pETIC vector were selected, and plasmids were prepared from these cells. Among the plasmids prepared in this manner, the plasmid into which the CREB fragment was inserted and the plasmid into which the kemptide fragment was inserted were designated "pETIC-ART" and "pETIC-Kempart, respectively.

Example 3: Production of a monitor protein

Monitor proteins comprising the phosphorylation sequence derived from either CREB or kemptide were produced using the above "pETIC-ART" or "pETIC-Kempart," respectively. The pETIC vector, the vector backbone for these plasmids, was prepared based on pET30a vector (Novagen), and has the functions of pET30a. Specifically, the pETIC vector contains a T7 promoter upstream of the insert region. Thus, expression of a target protein can be induced with IPTG in *E. coli* containing a T7 RNA polymerase gene downstream of a lacUV5 promoter, such as BL21 (DE3). The recombinant proteins expressed can easily be collected with Ni agarose because a histidine tag (His-tag) is added to the proteins. The monitor proteins were produced using "pETIC-ART" and "pETIC-Kempart" by the following procedure based on the above principle.

BL21(DE3) competent cells were transformed with the above "pETIC-ART" or "pETIC-Kempart" plasmid using the same method as mentioned above. After transformation, colonies formed on the kanamycin-containing agar medium were taken, inoculated into 3 ml of LB medium (kanamycin 50 μg/ml) , and incubated at 37°C overnight to prepare the preculture medium. This preculture medium (2 ml) was transferred into a 2-L flask containing 500 ml of the same LB medium, and cultured at 37°C until absorbance at 600 nm reached 0.6. IPTG was added thereto (concentration: 1 mM) when absorbance at 600 nm was 0.6, and culturing was further performed at 23°C overnight.

The cultured medium was centrifuged (5,000 rpm, 10 min, 4°C), and cells were harvested. The collected cells were suspended in 10 ml of a buffer (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 2 mM benzamidine, 1 mM PMSF). The cells in the resulting suspension were disrupted by sonication on ice five times by delivery of a 30-second pulse. The extract was mixed with NP-40 and imidazole at a final

concentration of 0.1% and 20 mM, respectively, stirred with a stirrer at 4°C, and centrifuged (10,000 rpm, 10 min) at 4°C to collect supernatant.

The collected supernatant was mixed with 500 µl of Ni-NTA-agarose (Qiagen) and stirred with a stirrer at 4°C for 60 min. After stirring, the Ni-NTA-agarose was washed with 2 ml of a binding buffer (50 mM Tris-HC1, pH 7.5, 150 mM NaCl, 2 mM benzamidine, 1 mM PMSF, 0.1% NP-40, 20 mM imidazole) four times.

After washing, the agarose was mixed with 1 ml of an elution buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 2 mM benzamidine, 1 mM -PMSF, 0.1% NP-40, 200 mM imidazole), stirred at 4°C with a stirrer for 30 min, and centrifuged to collect supernatant. This series of elution procedures was repeated twice. The collected eluate was mixed with glycerol at the final concentration of 20%, and stored at -80°C.

Ten microliters of the eluate collected in the above manner was subjected to 10% acrylamide-gel electrophoresis to confirm the presence of a monitor protein. After electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB). The results were shown in Fig. 2. In Fig. 2, pETIC-1 (Romeser, V. A. et al, J. Biol. Chem., 272: 13270-13274) is the pETIC vector into which a sequence encoding the Ca^{2+} calmodulin binding site was inserted. It was used as a positive control in the preparation of the recombinant protein (RSGFP - the calmodulin binding site - BSGFP). "IPTG-" and "IPTG+" indicate the absence and the presence, respectively, of induction with IPTG during the incubation.

As shown in Fig. 2, when expression is induced with IPTG, a single band is detected with each of "pETIC-ART"and "pETIC-Kempart." The sizes of these proteins were similar to those of the proteins expressed by pETIC-1, which is a positive control. Therefore, the proteins expressed here were expected to be the target

monitor proteins, namely RSGFP - CREB phosphorylation sequence - BSGFP 5 and RSGFP - kemptide phosphorylation sequence - BSGFP.

Example 4: Measurement of phosphorylation using γ-^{32}P

It was examined whether the phosphorylation sequences in the above two monitor proteins were phosphorylated in an *in vitro* system. This examination was conducted using A kinase as the kinase and [γ-^{32}P]ATP as the substrate according to the following procedure.

To prepare the reaction solution, 10 X kinase buffer (250 mM Hepes, pH 7.5, 100 mM $MgCl_2$, 10 mM DTT) (5 µl), A kinase (1 µl), [γ-^{32}P]ATP (0.5 µl), and sterilized water (43 µl) were mixed. To this mixture, each eluate (0. 5 µl) containing each monitor protein was added. The mixture containing the eluate was incubated at 30°C for 90 min to carry out phosphorylation reaction.

After the reaction, electrophoresis was conducted to determine whether each monitor protein was phosphorylated. As a pre-treatment for electrophoresis, the reaction solution after the reaction (50 µl) was mixed with 2 X sample buffer (50 µl) and boiled for 3 min. After boiling, the solution (20 µl) was subjected to electrophoresis on the 10% acrylamide gel. After electrophoresis, the gel was exposed to films to detect the signal of γ-^{32}P (Fig. 3).

As shown in Fig. 3, signals were detected both for the monitor protein derived from "pETIC-ART" and for the monitor protein derived from "pETIC-Kempart." Specificity of these signals was obvious by the fact that signals were not detected for pETIC-1-derived protein which did not contain any phosphorylation sequence. The respective positions of the signals detected for "pETIC-ART" and "pETIC-Kempart" corresponded to the size of the monitor proteins

expressed by the plasmids shown in Fig. 2. These results confirmed that the two monitor proteins produced in Example 3 maintained the phosphorylation sequences and that the sequences were phosphorylated.

Example 5: Measurement of phosphorylation by fluorescence change

One end of the phosphorylation sequence in the above two monitor proteins was ligated to RSGFP and the other to BSGFP. Fluorescence change due to interference of RSGFP and BSGFP at the each side by phosphorylation of the phosphorylation sequence in these two monitor proteins was examined.

As a reaction solution, 10 X kinase buffer (20 μl), ATP (100 mM) (2 μl), and sterilized water (168 μl) were mixed and the above monitor proteins (5 μg/μl) (10 μl) were added to this mixture. Moreover, to this reaction solution, 0, 1, 2, or 4 μl of A kinase were added.

The prepared reaction solution was incubated at 30°C for 30 min. Changes in the range of wavelengths absorbed at a range of wavelengths between 430 nm and 520 nm were measured in the incubated reaction solution with a fluorescence spectrophotometer (Nippon Bunnko). Figures 4, 5, and 6 show the results for fluorescence changes of "ART" (derived from pETIC-ART) , "Kempart- (derived from pETIC-Kempart), and pETIC-1 (negative control) derived recombinant proteins, respectively. In each of the above figures, different amounts of A kinase added to each reaction is shown as a different line.

As shown in Fig. 4, in "ART," a difference in the absorbance patterns was observed for reactions in which A kinase was not added and reactions in which A kinase was added. When A kinase was not added, the stronger absorbance was observed at 500 nm. As the amount of added A kinase increased, the absorbance at

450 nm was enhanced. This indicates that a conformational change was generated by phosphorylation of the CREB phosphorylation sequence, allowing RSGFP and BSGFP at either end of the CREB sequence to interfere with each other, emitting fluorescence.

In contrast, although phosphorylation of "Kempart" was confirmed using [γ-^{32}P]ATP in Example 3 above, no fluorescence change was observed in the above-described wavelength range (Fig. 5) as seen with the negative control (Fig. 6). From these results, it was expected that the phosphorylation sequence of kemptide was phosphorylated, but the phosphorylation was not accompanied by a conformational change.

Thus, it has been revealed that the above "ART" can be used for, for instance, detecting a phosphorylation reaction using the above-described fluorescence change as an indicator. An *in vivo* system can be constructed by introducing pETIC-ART, which expresses this "ART", into cells. Moreover, the above "ART" can be used not only for detecting a phosphorylation reaction, but also for screening kinases and screening accelerators and inhibitors of phosphorylation activity.

On the other hand, it was shown that there was a difference between the phosphorylation sequence of CREB and kemptide in terms of the presence and absence of conformational change at the time of phosphorylation. This finding indicates that RSGFP and BSGFP can be used for analyzing whether a conformational change occurs in phosphorylation sequences upon phosphorylation. Specifically, it was shown that a conformational change at the time of phosphorylation can be observed by inserting any phosphorylation sequence between RSGFP and BSGFP and by performing both phosphorylation measurements using [γ-^{32}P]ATP and fluorescence change.

Example 6: *In vivo* measurement of Phosphorylation

1) Structure and construction of pCEP4-ART

The pETIC-ART plasmid and the mammalian expression vector pCEP4 (Invitrogen) plasmid vector (Fig. 7) were digested with the restriction enzymes HindIII and XhoI and were electrophoresed on a 10% agarose gel. The target bands were collected using a Geneclean III kit. The collected DNA was dissolved in 10 μl of TE. Eight microliters of the DNA solution was mixed with 8 μl of solution I of DNA Ligation Kit Ver. 2 (Takara) to perform the ligation reaction at 16°C for 30 min. Eight microliters of the solution were mixed with 100 μl of JM109 *E. coli* competent cells, and the cells were transformed. The cells were spread on an ampicillin plate and cultured at 37°C overnight. Colonies were then taken and cultured in 3 ml of LB medium (ampicillin 50 μg/ml) at 37°C overnight. Two milliliters of the overnight cultured solution were added into 500 ml of LB medium (ampicillin 50 μg/ml) in a 2-liter flask, and cultured at 37°C overnight. The cultured *E. coli* was collected and the plasmid DNA (pCEP4-ART) was collected with Qiagen Tip (Qiagen).

2) Introduction of plasmid DNA into COS-7 cells

One microgram of pCEP4-ART plasmid DNA was added to 100 μl of serum-free DMEM medium. Four microliters of lipofectAMINE reagent (GIBCO BRL) were added to another 100 μl of serum-free DMEM medium. These two DMEM media were mixed together and allowed to stand for 30 to 40 min at room temperature. Then, medium in a glass-bottomed culture dish (MatTek) containing COS-7 cells, which had been passage-cultured the day prior to DNA introduction, was replaced with 800 μl of serum-free DMEM medium. The mixture of plasmid DNA

and lipofectAMINE reagent was added thereto and the cells were cultured for 3 hours. The medium was then replaced with 1 ml of DMEM medium containing 10% bovine serum, and culturing was performed for 2 days.

3) Imaging of phosphorylation

COS-7 cells transfected with pCEP-4 ART were spread on a cover slip (a diameter of 14 mm) of a glass-bottomed culture dish (MatTek). The cells were incubated at 30°C for 48 hours and rinsed twice with Ringer's buffer (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, Ph 7.4, and 5.6 mM glucose). The cells were then kept in the buffer in the dark at room temperature. Fluorescence intensity was measured with excitation light at 380 nm by detecting with 440 ± 20-nm and 520 ± 20-nm band pass filters using a 100 X oil immersion objective lens (OLYMPUS UV-Fluor), a Quantix CCD camera (Roper Scientific), and an IPLab Spectrum image processor (Scanalytics). Cells were exposed to the excitation light at 380 nm for a duration of 0.2 sec, and at exposure intervals of 20 sec. An image passed through the 440 ± 20 nm filter was detected, and after 5 seconds, an image passed through 520 ± 20 nm filter was detected. Cells were induced with dibutylyl-cAMP (db-cAMP), a cAMP analogue, by adding 500 µl of Ringer's buffer containing 10µM db-cAMP to ~~a~~ the culture dish containing 500-µl of Ringer's buffer.

4) Results

The intracellular distribution of "ART" in COS-7 cells was dispersed (Fig. 8A). The concentration of intracellular cyclic-adenosine monophosphate (cAMP) increased in the cells in the presence of db-cAMP, an analogue of cAMP, to activate A kinase. A fluorescence change in the cells was also measured in real time with a

cooled CCD camera (Fig. 8B). db-cAMP was added at 0 sec. Fluorescence intensity ratios in the cellular image indicate that values of fold induction (fluorescence intensity ratio) increased depending on the time after the addition of db-cAMP. The increase in fold induction was transmitted from the side of the cell membrane to the nucleus, coinciding with the phenomenon that an active form of A kinase moves from the cytoplasm to the nucleus after the increase of cytoplasmic cAMP. The average fold induction increased with time after db-cAMP stimulation. This indicates that "ART" can be used as phosphorylation monitor system in a living cell.

To examine whether "ART" is phosphorylated in COS-7 cells depending on A kinase, intracellular A kinase was inhibited with H-89, a selective inhibitor of A kinase. The experiment for db-cAMP stimulation was performed in the same manner as Fig. 8. The same experiment as described above was conducted except that the cells were pre-treated about for 2 hours with DMEM containing 10 μM H-89 and that the medium was then replaced with Ringer's buffer containing 10 μM H-89. Fluorescence change of "ART" in the cells was measured with a cooled CCD camera in real time (Fig. 9). Comparison of fluorescence intensity ratios in the region of a 10 μm X 10 μm range indicates that the fluorescence intensity ratio was low in the presence of H-89, showing that inhibition of A kinase activity can be observed in a living cell in real time.

As shown above, using the monitor proteins of the present invention, the system for monitoring phosphorylation *in vivo* was constructed. Using this system, phosphorylation can easily be monitored in an environment more similar to physiological conditions. Moreover, it was shown that the effects of inhibitors on kinase can easily be monitored using this system. Therefore, this system is useful for screening a compound which stimulates or inhibits phosphorylation.

Industrial Applicability

In the present invention, an analysis system was developed which uses no radioactive isotopes and is applicable to *in vivo* measurement of protein phosphorylation. The analysis system of the present invention can be used not only to detect a phosphorylation reaction, but also for screening a kinase and for screening a compound which stimulates or inhibits phosphorylation.